Characterization of human Smg5/7a: A protein with similarities to *Caenorhabditis elegans* SMG5 and SMG7 that functions in the dephosphorylation of Upf1

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ABSTRACT

Nonsense-mediated mRNA decay (NMD) in mammalian cells depends on phosphorylation of Upf1, an RNA-dependent ATPase and 5′-to-3′ helicase. Upf1 phosphorylation is mediated by Smg1, a phosphoinositol 3-kinase–related protein kinase. Here, we describe a human protein, which we call hSmg5/7a, that manifests similarity to *Caenorhabditis elegans* NMD factors CeSMG5 and CeSMG7, as well as two *Drosophila melanogaster* proteins that are also similar to the *C. elegans* NMD factors. Results indicate that hSmg5/7a functions in the dephosphorylation of Upf1. Furthermore, hSmg5/7a copurifies with Upf1, Upf2, Upf3X, Smg1, and the catalytic subunit of protein phosphatase 2A. We also demonstrate that Upf2, another factor involved in NMD, is a phosphoprotein. However, hSmg5/7a plays no role in the dephosphorylation of Upf2. These data indicate that hSmg5/7a targets protein phosphatase 2A to Upf1 but not Upf2. Results of Western blotting reveal that hSmg5/7a is mostly cytoplasmic in HEK293T cells.

Keywords: Human Smg5/7 protein; protein phosphatase 2A; phosphoprotein; Upf1 dephosphorylation; nonsense-mediated mRNA decay

INTRODUCTION

Eucaryotic cells eliminate mRNAs that prematurely terminate translation by using a mechanism called nonsense-mediated mRNA decay (NMD; Maquat 1995; Culbertson 1999; Frischmeyer and Dietz 1999; Hentze and Kulozik 1999; Hilleren and Parker 1999; Maquat and Carmichael 2001; Wilusz et al. 2001; Maquat 2002). Aberrant mRNAs that prematurely terminate translation can arise from routine abnormalities in gene expression, such as inefficient or inaccurate pre-mRNA splicing, or mutation. It behooves cells to eliminate these mRNAs, given their potential to encode proteins that are deleterious to cellular metabolism.

In NMD, a termination codon is generally identified as premature by its distance relative to the 3′-most exon-exon junction of the mRNA in which it resides: Termination codons located >50 to 55 nt upstream of this junction usually elicit NMD, whereas termination codons located <50 to 55 nt upstream of or downstream of this junction usually fail to elicit NMD (Cheng et al. 1994; Carter et al. 1996; Nagy and Maquat 1998; Thermann et al. 1998; Zhang et al. 1998a,b; Sun et al. 2000). The role of exon-exon junctions in NMD is attributed to an exon junction complex (EJC) that is deposited ~20 to 24 nt upstream of junctions as a consequence of pre-mRNA splicing (Kataoka et al. 2000; Le Hir et al. 2000a,b; McGarvey et al. 2000; Zhou et al. 2000). The EJC recruits the NMD factor Upf3/3X (also called Upf3a/b), which in turn recruits the NMD factor Upf2 (Lykke-Andersen et al. 2000, 2001; Mendell et al. 2000; Ishigaki et al. 2001; Kim et al. 2001; Serin et al. 2001; Lejeune et al. 2002). According to the current model, when translation termination is sufficiently premature, components of the termination complex, including but possibly not limited to the NMD factor Upf1, interact with EJC-bound Upf2 to trigger NMD.

Upf1, the most thoroughly characterized NMD factor, is a polysome-associated RNA-dependent ATPase and 5′-to-3′ helicase (Bhattacharya et al. 2000; Pal et al. 2001) that is required for NMD (Sun et al. 1998) and detected primarily

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Article and publication are at http://www.rnajournal.org/cgi/doi/10.1261/rna.2137903.

in the cytoplasm (Lykke-Andersen et al. 2000; Serin et al. 2001). Studies of Upf orthologs in *Saccharomyces cerevisiae* indicate that (1) Upf1 interacts with eukaryotic release factors (eRFs) 1 and 3; (2) Upf2 and Upf3 interact with eRF3 in a way that competes with the eRF1-eRF3 interaction; and (3) all three Upf proteins influence the translation termination efficiency of premature termination codon-containing transcripts (Czaplinski et al. 1998; Maderazo et al. 2000; Wang et al. 2001). Mammalian Upf1 is a phosphoprotein (Pal et al. 2001) that is targeted by the phosphoinositol 3-kinase (PIK)–related protein kinase Smg1 (Denning et al. 2001; Yamashita et al. 2001). Smg1 is named after its ortholog in *Caenorhabditis elegans* (Ce), CeSMG1, which phosphorylates CeSMG2, the *C. elegans* ortholog of mammalian Upf1 (Page et al. 1999). Data indicate that Upf1/CeSMG2 phosphorylation is critical for NMD: NMD is inhibited in mammalian cells by either overexpression of kinase-inactive Smg1 (Yamashita et al. 2001) or inhibition of Smg1 production using antisense RNA (K.M. Brumbaugh, D.M. Otterness, X. Li, L.E.M., and R.T. Abraham, unpubl. data), and NMD in *C. elegans* is inhibited by disruption of the *CeSMG1* gene (Page et al. 1999). Although nothing is known about Upf1 dephosphorylation, mutation of CeSMG5, CeSMG6, or CeSMG7 in *C. elegans*, each of which functions in CeSMG2 dephosphorylation, inhibits NMD (Page et al. 1999).

The present study aimed to gain insight into factors involved in mammalian Upf1 dephosphorylation. A database search for human cDNAs encoding proteins related to CeSMG5, CeSMG6, or CeSMG7 generated a protein that we named human (h) Smg5/7a. Consistent with a role in Upf1 dephosphorylation, Flag-tagged hSmg5/7a immunopurifies with Upf1, as well as Upf2, Upf3X, and Smg1. Flag-hSmg5/7a also immunopurifies with the catalytic subunit of protein phosphatase (PP) 2A and binds microcystin, indicating that it may be a PP2A subunit or target. In fact, transient expression of hSmg5/7a results in Upf1 dephosphorylation, indicating a role in targeting PP2A to Upf1. As demonstrated by two-dimensional gel electrophoresis, Upf2 is also a phosphoprotein but is insensitive to transient expression of hSmg5/7a. Western blotting indicates that Flag-hSmg5/7a is detected primarily in the cytoplasmic fraction of HEK293T cells.

RESULTS

Characterization of hSMG5/7a cDNA and encoded protein

The TBLASTN algorithm was used to analyze both the human genome (http://www.ncbi.nlm.nih.gov/blast/) and human cDNA (NR) databases, resulting in the identification of a cDNA (AB018275) encoding a protein having similarity to CeSMG5 and CeSMG7. In the absence of finding proteins more closely related to either CeSMG5 or CeSMG7, this protein was named human (h) Smg5/7a. hSmg5/7a consists of 1419 amino acids and has a predicted molecular weight of 163 kD and a pI of 7.5. According to analysis in silico, hSmg5/7a contains a putative PIN domain (Clissold and Ponting 2000) and three putative nuclear localization sequences (PSORT, http://psort.nibb.ac.jp/; Fig. 1A). The *hSMG5/7a* gene maps to the extremity of the small arm of chromosome 17 and contains 19 exons (http://www.kazusa.or.jp/huge/; Fig. 1B, which shows exons within cDNA). Two *Drosophila melanogaster* (Dm) cDNAs, CG8954 and CG6369, were also found to encode protein-containing domains related to both CeSMG5 and CeSMG7 by using the TBLASTN algorithm to analyze the *D. melanogaster* genome (http://www.fruitfly.org/blast/). Protein encoded by DmCG8954 cDNA consists of 1177 amino acids and has a predicted molecular weight of 136 kD (data not shown). Protein encoded by DmCG6369 cDNA consists of 949 amino acids and has a predicted molecular weight of 68 kD (data not shown).

Amino acids 721–816 of hSmg5/7a can be aligned to amino acids 136–214 of CeSMG7 and amino acids 279–375 of DmCG6369, and we have designated each region of each protein conserved region (C) 1 (Fig. 2). Additionally, amino acids 1241–1276 of hSmg5/7a can be aligned to amino acids 422–464 of CeSMG5, amino acids 765–815 of DmCG6369, and amino acids 1008–1055 of DmCG8954, and we have designated each region of each protein C2 (Fig. 2). Furthermore, amino acids 766–825 of hSmg5/7a and amino acids 285–358 of DmCG6369 contain regions similar to amino acids 130–163 and 164–197 of CeSMG7, both of which contain probable tetratricopeptide repeats (TPRs; Cali et al. 1999). TPRs form pairs of amphipathic α-helices that generally mediate protein-protein interactions (Das et al. 1998). Also, the putative PIN domain within hSmg5/7a amino acids 1246–1397 is conserved within amino acids 426–543 of CeSMG5 and has been proposed to manifest RNase activity (Clissold and Ponting 2000).

hSmg5/7a purifies with PP2Ac, Upf1, Upf2, Upf3X, and Smg1

Initially, immunopurifications were performed to identify proteins that interact either directly or indirectly with hSmg5/7a. To this end, pCI-neo-Flag-hSMG5/7a, a vector that expresses Flag-tagged hSmg5/7a, was transiently introduced into monkey kidney Cos cells. Two days later, total-cell protein was immunopurified by using anti-Flag antibody or, as a control for nonspecific immunopurification, anti-HA antibody. Subsequently, the presence of Flag-hSmg5/7a, as well as other proteins, was tested by using Western blotting.

Flag-hSmg5/7a was detected with anti-Flag antibody when protein was immunopurified with anti-Flag antibody but not anti-HA antibody (Fig. 3A), demonstrating the specificity of immunopurification. Because *C. elegans* mu-

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MAEGLERVRISASELRGILATLAPQAGSRENMKELKEARPRKDNRRPDLEIYKPGLSRLRNKPKIKEPPGSEEFKDEIVN :   80
DRDCSAVENGTQPVKDVCKELNNQEQNGPIDPENNRGQESFPRTAGQEDRSLKIIKRTKKPDLQIYQPGRRLQTVSKESA :  160
SRVEEEEVLNQVEQLRVEEDECRGNVAKEEVANKPDRAEIEKSPGGGRVGAAKGEKGKRMGKGEGVRETHDDPARGRPGS :  240
AKRYSRSDKRRNRYRTRSTSSAGSNNSAEGAGLTDNGQRRRRQDRTKERPPLKKQVSVSSTDSLDEDRIDEPDGLGPRRS :  320
SERKRHLERNWSGRGEGEQKTSAKEYRGTLRVTFDAEAMNKESPMVRSARDDMDRGKPDKGLSSGGKGSEKQESKNPKQE :  400
LRGRGRGILILPAHTTLSVNSAGSPESAPLGPRLLFGSGSKGSRSWGRGGTTRRLWDPNNPDQKPALKTQTPQLHFLDTD :  480
DEVSPTSWGDSRQAQASYYKFQNSDNPYYYPRTPGPASQYPYTGYNPLQYPVGPTNGVYPGPYYPGYPTPSGQYVCSPLP :  560
TSTMSPEEVEQHMRNLQQQELHRLLRVADNQELQLSNLLSRDRISPEGLEKMAQLRAELLQLYERCILLDIEFSDNQNVD :  640
QILWKNAFYQVIEKFRQLVKDPNVENPEQIRNRLLELLDEGSDFFDSLLQKLQVTYKFKLEDYMDGLAIRSKPLRKTVKY :  720
ALISAQRCMICQGDIARYREQASDTANYGKARSWYLKAQHIAPKNGRPYNQLALLAVYTRRKLDAVYYYMRSLAASNPIL :  800
TAKESLMSLFEETKRKAEQMEKKQHEEFDLSPDQWRKGKKSTFRHVGDDTTRLEIWIHPSHPRSSQGTESGKDSEQENGL :  880
GSLSPSDLNKRFILSFLHAHGKLFTRIGMETFPAVAEKVLKEFQVLLQHSPSPIGSTRMLQLMTINMFAVHNSQLKDCFS :  960
EECRSVIQEQAAALGLAMFSLLVRRCTCLLKESAKAQLSSPEDQDDQDDIKVSSFVPDLKELLPSVKVWSDWMLGYPDTW : 1040
NPPPTSLDLPSHVAVDVWSTLADFCNILTAVNQSEVPLYKDPDDDLTLLILEEDRLLSGFVPLLAAPQDPCYVEKTSDKV : 1120
IAADCKRVTVLKYFLEALCGQEEPLLAFKGGKYVSVAPVPDTMGKEMGSQEGTRLEDEEEDVVIEDFEEDSEAEGSGGED : 1200
DIRELRAKKLALARKIAEQQRRQEKIQAVLEDHSQMRQMELEIRPLFLVPDTNGFIDHLASLARLLESRKYILVVPLIVI : 1280
NELDGLAKGQETDHRAGGYARVVQEKARKSIEFLEQRFESRDSCLRALTSRGNELESIAFRSEDITGQLGNNDDLILSCC : 1360
LHYCKDKAKDFMPASKEEPIRLLREVVLLTDDRNLRVKALTRNVPVRDIPAFLTWAQVG                       : 1419
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B

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CCTGGCTGCGCGCGGCGGTGGCGGAGCCGCTACGGCTGTAGCAGCAGCCGCGAAGATGGCGGAAGGGCTGGAGCGTGTGCGGATCTCCGCGTCGGAGCTG :  100
CGCGGGATCCTGGCTACTCTGGCCCCGCAGGCCGGGAGCAGAGAAAACATGAAGGAATTAAAGGAGGCCAGGCCGCGCAAAGATAACAGGCGTCCAGATC :  200
TGGAAATCTATAAGCCTGGCCTTTCTCGGCTAAGGAACAAGCCCAAAATCAAGGAACCCCCTGGGAGTGAGGAATTCAAAGATGAAATTGTTAATGACCG :  300
AGATTGCTCTGCTGTTGAAAATGGTACACAGCCCGTTAAAGATGTCTGCAAGGAACTGAACAACCAAGAGCAGAATGGTCCTATAGACCCAGAAAATAAT :  400
CGGGGACAAGAATCCTTTCCTAGGACTGCTGGACAAGAGGATCGTAGTCTAAAAATTATCAAAAGAACAAAGAAACCCGACCTGCAGATCTATCAGCCTG :  500
GACGACGTTTGCAGACTGTTAGCAAAGAATCCGCCAGTCGGGTGGAGGAGGAAGAAGTCCTCAACCAGGTAGAACAACTGAGAGTAGAGGAAGATGAGTG :  600
TAGGGGAAATGTTGCGAAGGAGGAAGTTGCGAATAAACCAGACAGGGCCGAGATAGAAAAGAGCCCAGGTGGTGGGAGAGTAGGGGCTGCAAAAGGAGAA :  700
AAAGGAAAGAGGATGGGAAAAGGGGAGGGGGTGAGGGAAACCCACGACGACCCGGCCCGCGGGAGGCCGGGCTCCGCAAAGCGCTACTCCCGCTCAGACA :  800
AACGAAGGAATCGCTACCGCACGCGCAGCACCAGCTCAGCTGGCAGCAACAACAGCGCTGAGGGAGCTGGCCTGACGGATAATGGATGTCGCCGCCGCCG :  900
ACAGGATAGGACCAAGGAGAGGCCACCACTGAAGAAGCAAGTGTCTGTGTCCTCAACCGATTCCTTAGACGAGGACAGAATTGATGAGCCTGATGGATTA : 1000
GGACCCAGGAGAAGTTCAGAAAGGAAGAGACATTTAGAAAGAAACTGGTCTGGCCGTGGGGAGGGTGAGCAGAAAACCAGTGCTAAAGAATATCGAGGCA : 1100
CTCTTCGTGTCACTTTCGATGCAGAAGCCATGAACAAAGAGTCTCCCATGGTGAGGTCAGCCAGGGATGATATGGATAGAGGAAAGCCTGACAAAGGCTT : 1200
GAGCAGTGGGGGCAAAGGCTCTGAGAAGCAGGAGTCCAAAAACCCGAAACAAGAACTTCGGGGTCGTGGTCGTGGCATTCTGATTTTGCCTGCCCATACC : 1300
ACCCTATCTGTCAATTCAGCAGGTTCTCCAGAGTCCGCGCCTTTGGGACCTCGGCTTTTGTTTGGATCTGGTAGTAAGGGATCTCGGAGTTGGGGCCGTG : 1400
GAGGCACCACACGCCGATTGTGGGACCCAAACAATCCTGATCAGAAACCTGCTCTAAAGACTCAGACGCCCCAGCTACATTTCTTGGACACTGATGATGA : 1500
AGTCAGCCCTACATCTTGGGGTGACTCACGCCAGGCTCAGGCATCTTACTATAAGTTTCAAAACTCTGACAACCCCTATTATTACCCCCGGACACCAGGC : 1600
CCTGCCTCCCAGTATCCCTATACGGGCTATAACCCTCTACAGTACCCAGTGGGCCCTACGAATGGTGTGTACCCAGGGCCTTACTACCCAGGCTACCCGA : 1700
CTCCGTCAGGACAGTATGTGTGTAGCCCTCTACCTACCAGCACCATGAGTCCCGAGGAGGTAGAGCAGCACATGAGGAACCTGCAGCAACAGGAGCTGCA : 1800
CAGGCTTCTCCGGGTGGCTGACAACCAGGAACTGCAGCTCAGCAACCTGCTCTCCAGGGACCGCATCAGTCCGGAGGGCCTGGAGAAGATGGCGCAACTC : 1900
AGAGCTGAACTGCTGCAGCTATATGAGCGCTGTATTCTATTAGATATTGAGTTCTCTGATAATCAGAATGTGGATCAGATCCTGTGGAAGAATGCTTTCT : 2000
ATCAGGTGATTGAGAAGTTCAGGCAACTTGTCAAGGATCCGAATGTTGAGAACCCAGAACAGATTCGGAACAGACTTTTGGAGCTCTTGGATGAGGGTAG : 2100
TGACTTCTTTGATAGTTTGCTTCAGAAGCTGCAGGTTACTTACAAGTTCAAACTGGAAGACTACATGGATGGTCTTGCCATTCGCAGCAAGCCATTACGC : 2200
AAGACAGTAAAATATGCCTTGATCAGTGCCCAGCGATGCATGATATGCCAAGGAGATATTGCTAGGTACCGGGAGCAAGCCAGTGATACAGCGAATTATG : 2300
GGAAAGCACGCAGTTGGTACCTGAAGGCCCAGCACATTGCTCCCAAGAATGGGCGCCCCTATAACCAGTTGGCTTTGCTGGCAGTGTATACGAGGAGGAA : 2400
GCTTGACGCTGTCTATTACTATATGCGCAGTTTAGCTGCCAGCAACCCTATCCTGACTGCCAAGGAGAGTCTCATGAGCTTGTTTGAAGAGACCAAGCGG : 2500
AAGGCAGAACAGATGGAAAAGAAGCAACATGAGGAATTTGACCTGAGCCCTGACCAGTGGCGGAAAGGAAAGAAGTCTACTTTCCGGCATGTTGGAGATG : 2600
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CAGTACACAACTCCCAGCTGAAAGACTGCTTCTCGGAGGAGTGCCGCTCTGTGATCCAGGAACAAGCCGCAGCTCTGGGCTTGGCCATGTTTTCTCTACT : 3000
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GTCCCGGACCTGAAGGAGCTGCTCCCCAGTGTCAAAGTCTGGTCAGATTGGATGCTCGGCTACCCGGACACCTGGAATCCTCCTCCCACATCCCTGGATC : 3200
TGCCCTCGCATGTTGCTGTGGATGTATGGTCGACGCTGGCTGATTTCTGTAACATACTGACTGCAGTGAATCAGTCTGAGGTGCCACTGTACAAGGACCC : 3300
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GCTGGACGGCCTGGCCAAGGGGCAGGAGACAGACCACCGGGCTGGGGGCTACGCCCGTGTGGTACAAGAGAAGGCCCGCAAGTCCATCGAGTTCCTCGAG : 4000
CAGCGATTCGAGAGTCGGGACTCTTGCCTGCGAGCCCTGACCAGCCGTGGCAATGAACTCGAATCCATCGCCTTCCGCAGTGAGGACATCACTGGCCAGC : 4100
TGGGTAACAACGATGATCTCATCCTGTCCTGCTGCCTCCACTACTGCAAAGACAAGGCTAAGGACTTCATGCCCGCCAGCAAAGAGGAGCCAATCCGGCT : 4200
ACTGCGGGAGGTGGTGCTGTTGACGGATGACCGGAACCTGCGTGTGAAGGCGCTCACAAGGAATGTTCCTGTACGGGACATCCCAGCCTTCCTCACGTGG : 4300
GCCCAGGTGGGCTGAGGGAGCCACACTGGGGCCCCCCCCCCCCGTGGAACCGTTCCTGAAAGGCCACCAGGCGCCCAGTGTAGCACGGAAGATGCCCACG : 4400
TGCCTGAGCCACCAATCCACCCAGACAATAAACCATCCTCTTCCAACCCACGCCACGGCCATGCTGTGGGGACCTGCTCCTCACAGAGCCCCTCCCAAG : 4500
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GGTTCAGAGTTTCCGTCAGATGCAGTGAAATCACAGTTCCCTTTCATCTTCAGAACCTCTGTCGTGAATGTGTTCAAGAGGCTTTGGTTAAGTCAGGAAG : 4800
AAGTGCCCAGGGTGTGTGTCCCCAGTCTCCCTGAGGCCTGGACTCGCCCATGAACCCAAGTCGGCTTCTAGACAGCATGTCCCTAACAGCAGCCTGGGC : 4900
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CGTCCCCGTGGACAGTCATGAGTCTAGAGGAAAGGGGCATCTGGTCTCAGGCCCGTGCTCTCGGGTGGCCTCCACCTGCTCCCTTTCTCCTCACTGGCCT : 5100
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GCCTGAGGCATTCCAGTGCTGGGGCACCGTCGCCTAACCTGGTTTCTAGCTTTGCCCTCACTCCCCGGAAAAACTGACACTGACACAGGGGCCCTTTCCT : 5500
TGCCCCTTTAGCTGGTACCTCAGTGGGGAGGCTTCCTTACCAAGAATGAGTTCCTGAAACCCAGGGCCAGAGACAAGGACAACTTAGGGGAAGACGGGGT : 5600
TTTCGGTGGAGCCAGGGGCAAATCTTAATGGGACCAGTGGGGATACCCCAGAGCCCATGGCCTGACTGCACAGCCTGCCTGGAGGATGGGTGCGCAGCT : 5700
CTGCCCTCCCTGAGGCCCAGGACTATGCCAGAGCGATGGGGCACCGTGTAGGGGAGCCAAGGCCAGTAGTTTGGGGGTAGGAGTCCCCTAGAGTCTCAG : 5800
AAGACTGGGCTCTTTGGAGTACAGGGTCCCCGGCCTCTCCTTTAAGATTCTCTCCCCAGCTGGAAGGCCGATGACTGGGTGGTCGGGAGGGAGACCCAGC : 5900
TCTCCTTTCTGTCCCGTTTGCAGCACTGGTTTTGTTTCCTTAATAAATTTTTAGTTATGAAACAT                                   : 5965
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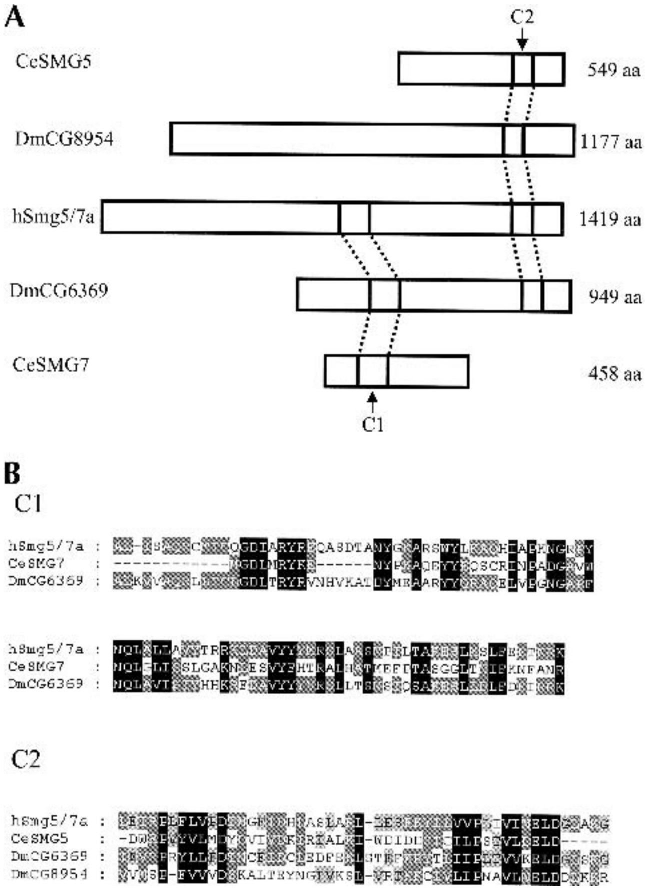
FIGURE 1. hSmg5/7a amino acid and hSMG5/7a cDNA sequences. (*A*) The 1419-aa sequence of hSmg5/7a is numbered to the *right*. Underlined amino acids constitute regions of a putative PIN domain. Three predicted nuclear localization sequences are boxed. (*B*) The 5965-nt sequence of hSMG5/7a cDNA (KIAA0732) is numbered to the *right*. The ATG initiation codon, TGA termination codon, and AATAAA polyadenylation sequence are boxed; exon-exon junctions are labeled with bold vertical lines; and the 5′- and 3′-untranslated regions may be incomplete.

tants that harbor defective *CeSMG5* or *CeSMG7* alleles are characterized by an abnormally high level of a phosphorylated isoform of CeSMG2 (Page et al. 1999), it is reasonable to think that CeSMG5 and CeSMG7 target a phosphatase to CeSMG2. Despite the limited sequence similarity of hSmg5/7a to either CeSMG5 or CeSMG7, the 36-kD catalytic subunit (c) of PP2A was detected in the anti-Flag antibody immunoprecipitate but not the anti-HA immunoprecipitate (Fig. 3A). Upf1, Upf2, and Upf3X also were detected in the anti-Flag antibody immunoprecipitate but not the anti-HA immunoprecipitate (Fig. 3A; Upf3 was too low in abundance to be detectable, as evidenced below). The presence of both eRFs was next assayed because (1) PP2A interacts with eRF1 (Andjelkovic et al. 1996); (2) *S. cerevisiae* Upf1 interacts with eRF1 and eRF3 (Czaplinski et al. 1998); and (3) *S. cerevisiae* Upf2 and Upf3 interact with eRF3 (Wang et al. 2001). However, neither eRF1 nor eRF3 was detected in the anti-Flag antibody immunoprecipitate (Fig. 3A; see below). To assay for the possibility that Smg1 associates with Flag-hSmg5/7a, it was necessary to analyze for exogenously expressed HA-tagged hSmg1 rather than endogenous Smg1 because the HA antibody is more sensitive than the available anti-Smg1 antibody. To this end, Cos cells were cotransfected with pCI-neo-Flag-hSMG5/7a and pCDNA-HA-hSMG1. Results indicate that both Flag-hSmg5/7a and HA-hSmg1 were immunopurified with anti-Flag antibody but not mouse IgG (Fig. 3A), indicating that hSmg5/7a and hSmg1 interact.

Microcystin purifies Flag-hSmg5/7a, Upf1, Upf2, Upf3, Upf3X, eRF1, and eRF3

Microcystin (MC)-Sepharose is often used to purify protein phosphatases and associated proteins (Moorhead et al. 1999). As an additional means to evaluate interactions of hSmg5/7a with other proteins, Flag-hSmg5/7a was transiently expressed in Cos cells, and total-cell protein was purified using MC-Sepha-



C

C1

Identity/ Similarity	hSmg5/7a	CeSMG7	DmCG6369
hSmg5/7a		28%/50%	49%/71%
CeSMG7			25%/48%
DmCG6369			

C2

Identity/ Similarity	hSmg5/7a	CeSMG5	DmCG6369	DmCG8954
hSmg5/7a		20%/53%	52%/76%	30%/61%
CeSMG5			28%/48%	22%/42%
DmCG6369				24%/50%
DmCG8954				

FIGURE 2. Alignment of human Smg5/7a to *Caenorhabditis elegans* SMG5 and SMG7 and *Drosophila melanogaster* CG8954 and CG6369. (*A*) hSmg5/7a, *C. elegans* (Ce) SMG5, CeSMG7, and *D. melanogaster* (Dm) CG8954, and DmCG6369 are diagrammed as horizontal bars. The number of constituent amino acids (aa) is provided to the *right* of each bar. Conserved regions 1 (C1) and 2 (C2) are boxed and aligned with dashes. (*B*) Amino acids 721–816 of hSmg5/7a, 136–214 of CeSMG7, and 279–375 of DmCG6369 are aligned at C1; amino acids 1241–1276 of hSmg5/7a, 422–464 of CeSMG5, 765–815 of DmCG6369, and 1008–1055 of DmCG8954 are aligned at C2. White letters in black boxes specify amino acids that are either identical or similar among all compared proteins. White letters in gray boxes specify amino acids that are either identical or similar among only two (C1) or three (C2) compared proteins. Black letters in gray boxes specify amino acids that are either identical or similar between only two (C2) compared proteins. (*C*) Percent (%) identity/similarity between proteins within C1 or C2.

rose and, as a control, unconjugated Sepharose. PP2Ac along with Flag-hSmg5/7a, Upf1, Upf2, Upf3, Upf3X, eRF1, and eRF3 were purified by using MC-Sepharose but not Sepharose alone (Fig. 3B). Our inability to detect eRF1 or eRF3 in the immunopurification of Flag-hSmg5/7a with anti-Flag antibody (Fig. 3A) suggests that neither release factor interacts directly with hSmg5/7a (see Discussion).

Overexpression of hSmg5/7a reduces the phosphorylation of hUpf1

The finding that Flag-hSmg5/7a copurifies with PP2Ac indicates that it could function in Upf1 dephosphorylation analogously to the way CeSMG5 and CeSMG7 function in CeSMG2 dephosphorylation. To examine this possibility, pCI-neo-Flag-hSMG5/7a or, as a control, pCI-neo was transiently introduced into HEK293T cells together with pCI-neo-Flag-hUPF1 (Pal et al. 2001). Two days later, total-cell protein was purified, and the ratio of Flag-hSmg5/7a to Flag-hUpf1 was determined by using Western blot analysis and anti-Flag antibody to be 1:10 (Fig. 4A). Flag-hUpf1 (together with Flag-hSmg5/7a) was then immunopurified with anti-Flag antibody, and the effect of Flag-hSmg5/7a on the phosphorylation status of Flag-hUpf1 was analyzed by using two-dimensional gel electrophoresis and Western blotting using anti-Flag antibody (Pal et al. 2001). When pCI-neo was expressed, Flag-hUpf1 was detected as multiple spots, each of which is potentially a different phosphorylated isoform (Fig. 4B; Flag-hSmg5/7a was not detected under the conditions used), as expected from previous results (Pal et al. 2001). Exposure to λ-phosphatase (λ-PPase) reduced or eliminated the more acidic isoforms, consistent with the isoforms being phosphoproteins. Notably, the degree of Flag-hUpf1 phosphorylation was also significantly reduced when pCI-neo-Flag-hSMG5/7a was ex-

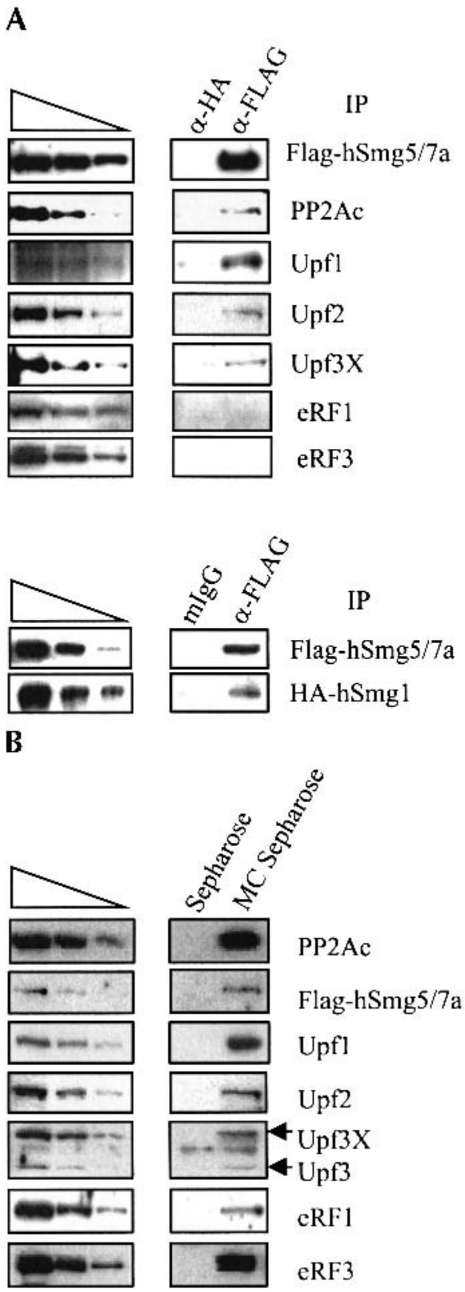


FIGURE 3. Flag-hSmg5/7a associates with PP2Ac, Upf1, Upf2, Upf3X, and hSmg1, but not with eRF1 and eRF3. (*A*) Flag-hSmg5/7a and, when specified, HA-hSmg1 were transiently expressed in Cos cells, and total-cell lysate was purified by using anti-Flag antibody, which purified 26% (*top*) or 3% (*bottom*) of cellular Flag-hSmg5/7a, or anti-HA or mouse IgG, each of which controlled for nonspecific immunopurification. (*B*) Alternatively, lysate was purified by using microcystin (MC)-Sepharose, which purified 50% of cellular PP2Ac, or unconjugated Sepharose, which controlled for nonspecific binding to Sepharose alone. Purified material was then analyzed by Western blotting with the specified antibodies, which consisted of those against PP2Ac, Upf1, Upf2, Upf3/3X, eRF3, eRF1, Flag (to detect Flag-hSmg5/7a), and HA (to detect HA-hSmg1).

pressed (Fig. 4B). We conclude that hSmg5/7a functions in hUpf1 dephosphorylation.

Overexpression of hSmg5/7a does not reduce the phosphorylation of hUpf2

Just as mammalian Upf1 is a phosphoprotein, mammalian Upf2 could be a phosphoprotein. To test this possibility and simultaneously assay for effects of overexpressing hSmg5/7a, T7-tagged hUpf2 was transiently produced in HEK293T cells together with pCI-neo or pCI-neo-Flag-hSMG5/7a. The expression of T7-hUpf2 and Flag-hSmg5/7a was confirmed by using Western blotting with, respectively, anti-T7 antibody and anti-Flag antibody (Fig. 5A). However, the ratio of the two proteins was undeterminable because the reactivity of each antibody is undoubtedly different. T7-hUpf2 was then immunopurified by using anti-T7 antibody either with or without exposure to λ-PPase. Two-dimensional gel electrophoresis and Western blotting using anti-T7 antibody revealed that, like Flag-hUpf1, T7-hUpf2 was detected as multiple spots when pCI-neo was expressed (Fig. 5B; Flag-hSmg5/7a did not enter the gel under the conditions used). At least the majority of these spots correspond to phosphoprotein as evidenced by their reduction or elimination on exposure to λ-PPase (Fig. 5B). Therefore, T7-hUpf2 is a phosphoprotein. However, the degree of T7-hUpf2 phosphorylation was unaffected when pCI-neo-Flag-hSMG5/7a was expressed (Fig. 5B), indicating that Flag-hSmg5/7a does not target T7-hUpf2 for dephosphorylation.

Flag-hSmg5/7a is located primarily in the cytoplasmic fraction of HEK293T cells

Insight into protein function often derives from information on where the protein localizes in cells. We rationalized that near-normal levels of Flag-hSmg5/7a should localize as does endogenous hSmg5/7a. Therefore, HEK293T cells were transiently transfected with pCI-neo-Flag-hSMG5/7a so that the level of Flag-hSMG5/7a RNA was only 1.6-fold higher than the level of endogenous SMG5/7a RNA (Fig 6A). Nuclear and cytoplasmic fractions were generated under conditions in which PLC-γ was detected by Western blotting primarily in the cytoplasmic fraction (Fig. 6B).

Flag-hSmg5/7a was detected primarily in the cytoplasmic fraction (Fig. 6B). eRF1 and eRF3 were also detected primarily but not exclusively in the cytoplasmic fraction (Fig. 6B), consistent with the recent finding that a fraction of eRF3 localizes to transcriptionally active loci, as well as the nucleolus (Brogna et al. 2002). For reasons not understood, Upf1 was detected primarily in the nuclear fraction (Fig. 6B). This result is surprising because results of in situ hybridizations indicate that Upf1 endogenous to HeLa cells, and Flag-hUpf1 transiently expressed in HeLa cells are mostly cytoplasmic (Lykke-Andersen et al. 2000; Serin et al.

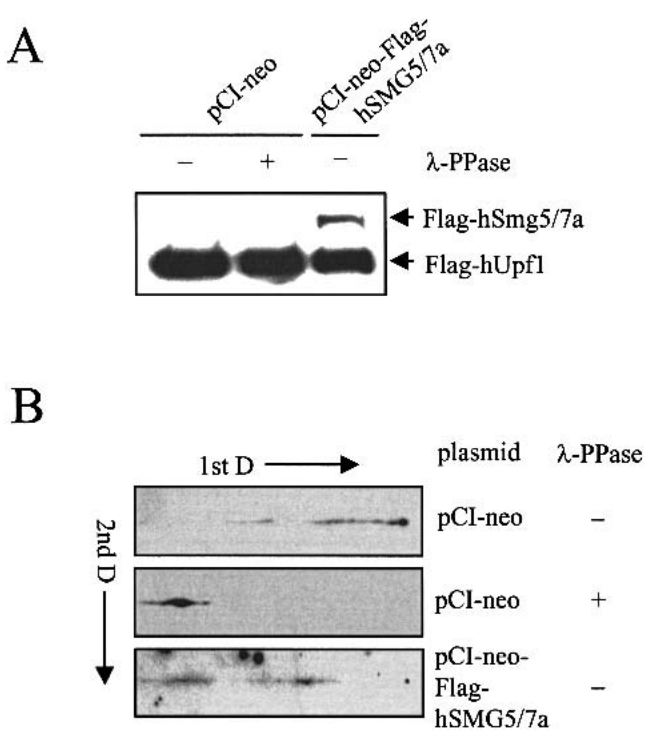


FIGURE 4. Overexpression of hSmg5/7a reduces the level of Upf1 phosphorylation. HEK293T cells were transiently transfected with pCI-neo-Flag-hUPF1 and either pCI-neo-Flag-hSMG5/7a or, as a control, pCI-neo. Total-cell lysates were purified by using anti-Flag antibody and incubated with or without λ-phosphatase (λ-PPase). (*A*) A fraction of each lysate prior to immunopurification was analyzed by Western blotting and anti-Flag antibody in order to quantitate the expression of Flag-hSmg5/7a and Flag-hUpf1 in each sample. (*B*) Comparable amounts of immunopurified Flag-hUpf1 were then subjected to two-dimensional gel electrophoresis under conditions that precluded detection of Flag-hSmg5/7a, and Flag-hUpf1 was localized by blotting with anti-Flag antibody.

2001). However, recent studies using immunofluorescence and confocal microscopy have demonstrated that the endogenous Upf1 of HeLa cells accumulates in the nuclei when CRM1-mediated nuclear export is inhibited by using leptomycin B (Mendell et al. 2002). Upf2 was detected primarily but not exclusively in the cytoplasmic fraction (Fig 6B). Until now, immunofluorescence has demonstrated that Upf2 endogenous to HeLa cells, and T7-hUpf2 transiently expressed in HeLa cells are detected essentially exclusively in the cytoplasm, concentrated around the cytoplasmic side of the nuclear envelope (Lykke-Andersen et al. 2000; Serin et al. 2001), and HA-hUpf2 (also called RENT2) has been deemed to be exclusively cytoplasmic by using immunofluorescence coupled with confocal microscopy (Mendell et al. 2000).

DISCUSSION

The present study provides the first description of hSmg5/7a (Fig. 1A), which has limited and localized identity and similarity to both CeSMG5 and CeSMG7 (Fig. 2). We show that hSmg5/7a functions in the dephosphorylation of Upf1, as would be predicted if it were a functional ortholog to the *C. elegans* proteins (Fig. 4). Additionally, we show that Upf2 is a phosphoprotein but is not subject to hSmg5/7a-mediated dephosphorylation (Fig. 5).

Consistent with a role in Upf protein dephosphorylation, hSmg5/7a interacts with PP2Ac (Fig. 3). The PP2As are a large and diverse family of multimeric serine/threonine phosphatases that function to regulate many cellular processes in eukaryotes (Millward et al. 1999; Janssens and Goris 2001). PP2A contains two subunits in addition to the catalytic (C) subunit: a structural or scaffolding (A) subunit and a regulatory (B) subunit, the latter of which can influence phosphatase activity, substrate specificity, and subcellular location. Holoenzyme interacting proteins include substrates. Therefore, the finding that hSmg5/7a immunopurifies with Upf proteins, coupled with the finding that overexpression of hSmg5/7a causes Upf1 protein dephosphorylation, indicates that hSmg5/7a delivers PP2A to Upf1

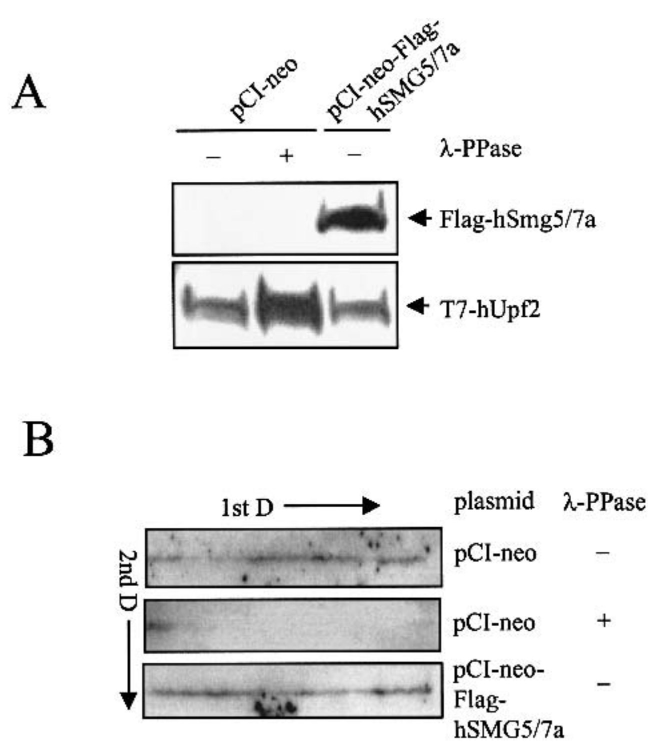


FIGURE 5. hUpf2 is a phosphoprotein, but overexpression of hSmg5/7a does not reduce the level of hUpf2 phosphorylation. HEK293T cells were transiently transfected with pCI-neo-T7-hUPF2 and either pCI-neo-Flag-hSMG5/7a or, as a control, pCI-neo. Total-cell lysates were purified by using anti-T7 antibody and incubated with or without λ-phosphatase (λ-PPase). (*A*) A fraction of each lysate was analyzed by Western blotting and anti-T7 or anti-Flag antibody in order to demonstrate the presence of T7-hUpf2 and Flag-hSmg5/7a. (*B*) Comparable amounts of immunopurified T7-hUpf2 were then subjected to two-dimensional gel electrophoresis, and T7-hUpf2 was localized by Western blotting and anti-T7 antibody.

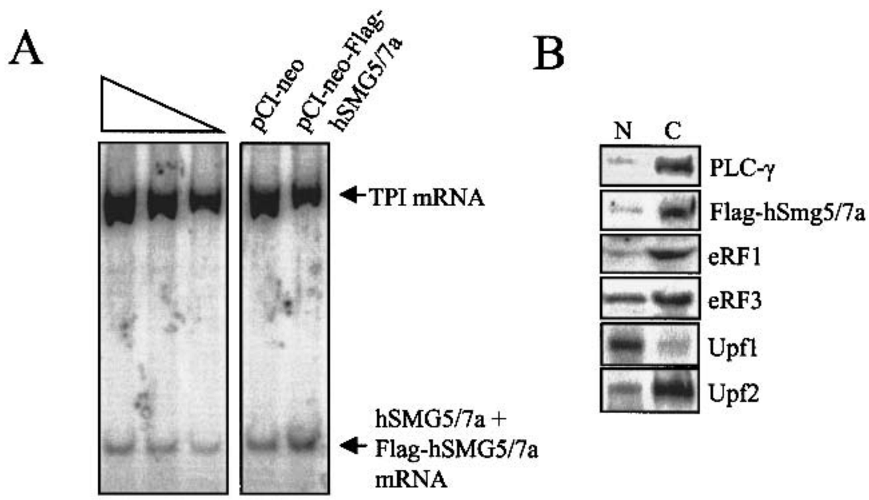


FIGURE 6. hSmg5/7a localizes primarily to the cytoplasmic fraction of HEK293T cells. Flag-hSMG5/7a was transiently expressed in HEK293T cells. Subsequently, RNA was purified from total-cell lysates, and protein was purified from nuclear and cytoplasmic fractions. (*A*) RT-PCR was used to determine the level at which Flag-hSmg5/7a was expressed relative to endogenous Smg5/7a. Considering a transfection efficiency of 90% (data not shown), the combined levels of endogenous and exogenous hSMG5/7a mRNA in transfected cells was determined to be only 2.6-fold of the level of endogenous hSMG5/7a mRNA in untransfected cells. (*B*) Western blotting using antibodies against PLC-γ, Flag (to detect Flag-hSmg5/7a), eRF1, eRF3, Upf1, and Upf2 was used to determine the cellular distribution of the various proteins.

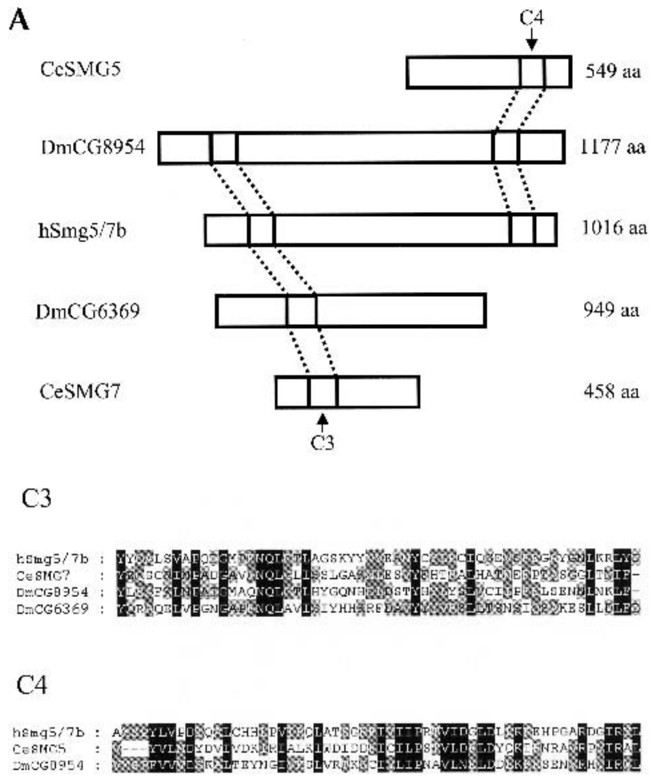
similarly to the way that eRF1 has been proposed to deliver PP2A to polysomes (Lechward et al. 1999). Along similar lines, co-immunopurification of hSmg5/7a and the PIK-related protein kinase hSmg1 (Fig. 3) probably reflects that both effectors target Upf1. Alternatively or additionally, hSmg5/7a could target hSmg1, in keeping with the observation that the majority of PP2A substrates identified to date are protein kinases (Lechward et al. 1999). It is also possible that hSmg5/7a is itself a substrate of PP2A.

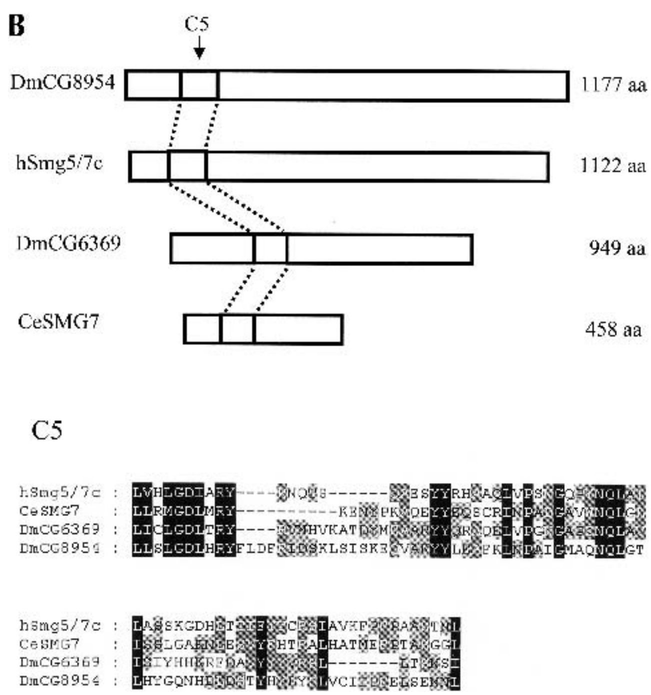
eRF1 and eRF3 were not detected in the immunopurification of Flag-hSmg5/7a using anti-Flag antibody (Fig. 3A). In *S. cerevisiae*, both eRF1 and eRF3 interact with Upf1 (Wang et al. 2001). It has been proposed that dissociation of eRF1 from eRF3 allows binding of Upf2 and Upf3 to a putative surveillance complex through interactions with eRF3 (Wang et al. 2001). In fact, the dissociation of eRF3 from Upf1 activates the Upf1 ATPase activity (Wang et al. 2001). If Upf1 interacts with eRF1 and eRF3 in mammalian cells, then our inability to detect either release factor in association with Flag-hSmg5/7a suggests that hSmg5/7a binds to Upf1 after eRF1 and eRF3 dissociate from Upf1.

Flag-hSmg5/7a is mostly cytoplasmic in HEK293T cells when produced from a transiently introduced plasmid that generates Flag-hSMG5/7a RNA at only 1.6-fold the level of endogenous hSMG5/7a RNA (Fig. 6). Notably, this finding does not preclude the possibility that Flag-hSmg5/7a is imported into nuclei, as would be predicted from a computer-based analysis demonstrating the presence of three nuclear localization sequences (Fig. 1A). In fact, Flag-hSmg5/7a is mostly nuclear in Cos cells, but under conditions in which the exact level of expression relative to endogenous hSmg5/7a could not be accessed because primer pairs used to amplify hSMG5/7a transcripts did not cross-react with Cos-cell RNA (data not shown).

A database search revealed two other human proteins that are similar to CeSMG5 and CeSMG7 (Fig. 7). We call these proteins hSmg5/7b (KIAA1089) and hSmg5/7c (KIAA0250). The *hSMG5/7b* gene maps to chromosome 1 and encodes a protein of 1016 amino acids with a predicted molecular weight of 114 kD and a putative PIN domain that spans amino acids 873–972. The *hSMG5/7c* gene also maps to chromosome 1 and encodes a protein of at least 1122 amino acids (to date, only a partial sequence is available). An interspecies comparative analysis revealed that amino acids 853–924 of hSmg5/7b can be aligned to amino acids 425–480 of CeSMG5 and amino acids 1009–1081 of DmCG8954 (Fig. 7A). Amino acids 173–257 of hSmg5/7b can be aligned to amino acids 130–208 of CeSMG7, amino acids 286–370 of DmCG6369, and amino acids 211–298 of DmCG8954 (Fig. 7A). Additionally, amino acids 186–257 of hSmg5/7c can be aligned to amino acids 133–208 of CeSMG7, amino acids 288–361 of DmCG6369, and amino acids 213–299 of DmCG8954 (Fig. 7B). An intraspecies comparative analysis revealed that amino acids 636–823 of hSmg5/7a, 65–268 of hSmg5/7b, and 84–274 of hSmg5/7c can be aligned with 21% identity and 45% similarity between hSmg5/7a and hSmg5/7b, 25% identity and 47% similarity between hSmg5/7b and hSmg5/7c, and 28% identity and 48% similarity between hSmg5/7a and hSmg5/7c (data not shown). However, we were unable to identify a conserved functional motif within these regions.

The presence of human proteins that contain regions related to both CeSMG5 and CeSMG7 indicates that the *C. elegans* proteins may function as a heterodimer. In fact, CeSMG5 interacts with CeSMG7 (Cali et al. 1999), and the interaction is direct (A. Grimson and P. Anderson, pers. comm.). The finding that mutation of either *C. elegans* protein abolishes NMD (Pulak and Anderson 1993) and prevents the dephosphorylation of CeSMG2 (Page et al. 1999) reinforces the idea of cooperative function in NMD. Future studies aim to assess the functional significance of hSmg5/7b and hSmg5/7c to the extent of Upf protein phosphorylation. We also aim to determine if all three hSmg5/7 proteins function in NMD.





C

C3

Identity/ Similarity	hSmg5/7b	CeSMG7	DmCG8954	DmCG6369
hSmg5/7b		28%/54%	35%/59%	31%/54%
CeSMG7			28%/50%	29%/59%
DmCG8954				28%/50%
DmCG6369				

C4

Identity/ Similarity	hSmg5/7b	CeSMG5	DmCG8954
hSmg5/7b		27%/46%	37%/62%
CeSMG5			31%/51%
DmCG8954			

C5

Identity/ Similarity	hSmg5/7c	CeSMG7	DmCG6369	DmCG8954
hSmg5/7c		27%/57%	35%/52%	30%/43%
CeSMG7			30%/53%	28%/46%
DmCG6369				30%/56%
DmCG8954				

FIGURE 7. Alignment of hSmg5/7b and hSmg5/7c to CeSMG5 and CeSMG7 and DmCG8954, and DmCG6369. (*A*, *B*) hSmg5/7b or hSmg5/7c, CeSMG5, CeSMG7, DmCG8954, and DmCG6369 are diagrammed as horizontal bars. The number of constituent amino acids (aa) is provided to the *right* of each bar. Conserved regions 3 (C3), 4 (C4), and 5 (C5) are boxed and aligned with dashes. Amino acids 173–257 of hSmg5/7b, 130–208 of CeSMG7, 286–370 of DmCG6369, and 211–298 of DmCG8954 can also be aligned at C3. Amino acids 853–924 of hSmg5/7b, 1009–1081 of DmCG8954, and 425–480 of CeSMG5 can be aligned at C4. Amino acids 186–257 of hSmg5/7c can be aligned with amino acids 133–208 of CeSMG7, 288–361 of DmCG6369, and 213–299 of DmCG8954 at C5. White letters in black boxes, white letters in gray boxes, and black letters in gray boxes are as in Figure 1B. (*C*) Percent (%) identity/similarity between proteins within C3, C4, and C5.

MATERIALS AND METHODS

Plasmid constructions

pCI-neo-Flag-hSMG5/7a was generated in two steps. First, the 6-kbp *Not*I-*Not*I fragment containing the full-length coding region of hSmg5/7a was inserted in the *Not*I site of pCI-neo (Invitrogen). Second, DNA encoding the Flag epitope was PCR-generated by using Vent polymerase, pBS-hSMG5/7a, and primers 5′-*GCTAGC*TCGAGACCGGTGCCACCATGGACTACAAAGACGATGACGACAAGGCGGAAGGGCTGGAGCGTGTGCGGATC-3′ (sense, where the *Nhe*I site is italicized, and the Flag coding region is underlined) and 5′-TTTAAACCC*GGCCTGCGGGGCC*AGAGTAGCCAGGATCCCGCGC-3′ (antisense, where the *Sfi*I site is italicized). The PCR product was digested with *Nhe*I and *Sfi*I and inserted into the *Nhe*I and *Sfi*I sites of the plasmid generated in the first step.

Cell culture and transfection

Monkey kidney Cos-7 and HEK293T cells were cultured in DMEM (GIBCO BRL) with 10% fetal calf serum. Cells were transfected with plasmids by using LipofectAMINE 2000 (Life Technologies).

Cell fractionation, lysis, and immunopurification or microcystin binding

Cos cells (6×10^7 to 7×10^7) were lysed, or 293T cells (3×10^6 to 4×10^6) were fractioned into nuclei and cytoplasm, and protein complexes were immunopurified essentially following the method of Ishigaki et al. (2001). For immunopurification, briefly, Cos-cell lysates were cleared using protein G-agarose beads (Boehringer Mannheim) or Sepharose beads (Sigma) and then incubated in the presence of mouse IgG (Sigma), anti-HA antibody (Roche), or anti-Flag antibody (Sigma) for 90 min at 4°C. Subsequently, protein G-agarose beads and 2 mg of yeast tRNA (Sigma) or microcystin-conjugated Sepharose beads (Upstate Technology) were added, and the incubation was continued for an additional 60 min or overnight at 4°C. The beads were washed five times with NET-2 buffer or lysis buffer (10 mM HEPES at pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.1% NP-40, 1 mM PMSF, and 2 mM benzamidine) and suspended in 50 μL of SDS sample buffer (Ishigaki et al. 2001). Immunopurified material was then analyzed by using Western blotting.

Western blotting

Immunopurified proteins or proteins in nuclear and cytoplasmic fractions were electrophoresed in polyacrylamide (6% for Flag-hSmg5/7a, HA-Smg1, Upf1, Upf2 or PLC-γ; 10% for PP2Ac, Upf3, Upf3X, eRF1, or eRF3). Proteins were transferred to Hybond ECL nitrocellulose (Amersham) and probed with antibody against Flag, HA, PP2Ac (BD Biosciences), one of the Upf proteins (Ishigaki et al. 2001; Serin et al. 2001), eRF1 or eRF3 (gifts from B. Hemmings, The Friedrich Miescher-Institut, Basel, Switzerland), PLC-γ (a gift of R.T. Abraham, The Burnham-Institute, San Diego, CA), or T7 (Novagen). Reactivity to each antibody was detected by using a 1:5000 dilution of horseradish peroxidase–conjugated donkey anti-rabbit antibody (Amersham; for Upfs, eRFs, or PLC-γ) or horseradish peroxidase–conjugated sheep anti-mouse antibody (Amersham; for Flag-hSmg5/7a, HA-Smg1, PP2Ac, or T7). Reactivity of the secondary antibody was visualized by using SuperSignal West Pico or Femto solution (Pierce).

Two-dimensional gel analysis

The two-dimensional analysis of proteins was performed essentially according to the method of Pal et al. (2001). HEK293T cells (3×10^6 to 4×10^6 for the analysis of Flag-hUpf1, or 2×10^7 for the analysis of T7-hUpf2) were lysed in extraction buffer (Pal et al. 2001) in the presence or absence of 0.25 mM Na-o-vanadate, 10 mM NaF, and 10 nM okadaic acid. Lysates were incubated with anti-Flag or anti-T7 antibody for 2 h at 4°C and, subsequently, for 2 h with protein G-agarose. The beads were washed once with λ-phosphatase buffer (New England BioLabs) and incubated with or without 400 U of λ-phosphatase (New England BioLabs) for 30 min at 30°C. The beads were then washed once with extraction buffer, and immunopurified material was eluted in sample buffer (0.1 M Tris-HCl at pH 6.8, 2% Triton X-100, 20% glycerol, 10% 2-mercaptoethanol). For the first dimension, isoelectric focusing was performed by using Bio-lytes (pH 3 to 10 and pH 5 to 7 for Flag-hUpf1; pH 4 to 6 for T7-hUpf2). For the second dimension, separation according to molecular mass was performed by electrophoresis in 6% polyacrylamide. Proteins were transferred to Hybond ECL nitrocellulose and probed with anti-Flag antibody conjugated to peroxidase or anti-T7 antibody. Reactivity of anti-T7 antibody was detected by using horseradish peroxidase–conjugated sheep anti-mouse antibody. Reactivity of the primary or secondary antibody was visualized using SuperSignal West Femto solution.

RT-PCR

TPI and hSMG5/7a mRNAs were analyzed by RT-PCR as described (Iskigaki et al. 2001). TPI mRNA was amplified using primers 5′-TGACCTTCAGCGCCTCGG-3′ (sense) and 5′-CTCCGAGTCCCTCTGCC-3′ (antisense). hSMG5/7a mRNA was amplified using primers 5′-GGCAAAGGCTCTGAGAAGC-3′ (sense) and 5′-CCGAGGTCCCAAAGGCG-3′ (antisense). The simultaneous analysis of serial dilutions of RNA ensured that RT-PCR was quantitative. RT-PCR products were quantitated by PhosphorImaging (Molecular Dynamics).

ACKNOWLEDGMENTS

We thank Linda McKendrick, Xiaojie Li, Brian Wadzinski, and Chris Ponting for helpful discussions; Bob Abraham for pCDNA-HA-hSMG1 and anti-PLC-γ antibody; Brian Hemmings for anti-eRF1 and anti-eRF3 antibodies; and Fabrice Lejeune for comments on the manuscript. This work was supported by Public Health Service grants to L.E.M. from the NIH.

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Received September 10, 2002; accepted October 8, 2002.

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